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Species identification of modern and archaeological shark and ray skeletal tissues using collagen peptide mass fingerprinting

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Introduction: Elasmobranchs, such as sharks and rays, are among the world's most endangered vertebrates, with over 70% loss in abundance over the past 50 years due to human impacts. Zooarchaeological baselines of elasmobranch diversity, distribution, and exploitation hold great promise for contributing essential historical contexts in the assessment of contemporary patterns in their taxonomic diversity and vulnerability to human-caused extinction. Yet, the historical ecology of elasmobranchs receives relatively less archaeological attention compared to that of ray-finned fishes or marine mammals, largely due to issues of taxonomic resolution across zooarchaeological identifications.

Methods: We explore the use of Zooarchaeology by Mass Spectrometry (ZooMS) for species identification in this unstudied group, using an archaeological case study from the marine environments of the Florida Keys, a marine biodiversity hotspot that is home to an array of elasmobranch species and conservation efforts. By comparison with 39 modern reference species, we could distinguish 12 taxa within the zooarchaeological assemblage from the Clupper archaeological site (Upper Matecumbe Key) that included nine sharks, two rays and a sawfish.

Results and discussion: The results indicate that, through additional complexity of the collagen peptide mass fingerprint, obtained due to the presence of the cartilaginous type II collagen, ZooMS collagen peptide mass fingerprinting provides exceptionally high taxonomic resolution in this group, yielding species-level identifications in all cases where sufficient reference material was used. This case study also highlights the added value of ZooMS for taxa that are more difficult to distinguish in zooarchaeological analyses, such as vertebrae of the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*) and the hammerhead sharks (*Sphyrna* spp.) in the Florida Keys. Therefore, the application of collagen peptide mass fingerprinting to elasmobranchs offers great potential to improve our understanding of their archaeological past and historical ecology.

KEYWORDS

collagens, cartilage, ZooMS, species identification, Florida Keys, proteomics

Introduction

Elasmobranchs (sharks, skates, and rays) are essential to the health of marine ecosystems, with many (particularly sharks) often playing vital roles as keystone taxa and apex predators, helping to maintain species diversity and ecosystem health (Heithaus et al., 2007; Motivarash Yagnesh et al., 2020). Today, despite having existed for over 400 million years, elasmobranchs are among the world's most endangered vertebrate groups due to human impacts, with over 70% loss in abundance over the past 50 years (Pacoureau et al., 2021). This has been particularly devastating to shark populations, whose life histories are characterized by low fecundity, slow growth, late maturity, and relatively long-life spans, all of which makes them particularly vulnerable to population decline via anthropogenic threats such as over-fishing, habitat degradation, and accelerated climate change (Dulvy et al., 2021; Field et al., 2009; Sherman et al., 2023). Moreover, increases in shark exploitation are also linked to declines in popular bony fishes available for harvest (Camhi et al., 1998), as well as rising demand within the international shark fin trade network (Worm et al., 2024).

The historical ecology of elasmobranchs, and especially sharks, demonstrates that humans have engaged with and harvested taxa across the world for millennia (e.g., the Mediterranean [Giovos et al., 2021; Mojetta et al., 2018]; the North Sea [Bom et al., 2022]; the Gulf of Mexico [Martínez-Candelas et al., 2020]; Southeast Asia [Boulanger, 2023; Boulanger et al., 2021]). Utilizing historical perspectives of shark diversity and harvest patterns, garnered through sources such as oral histories (including traditional ecological knowledge, and indigenous traditional ecological knowledge), ethnohistoric texts, art, photography, and more recent (e.g., decade-scale) survey data, make it clear that the worldwide decline in taxa has been driven by human overfishing, with particular emphasis on loss over the past century (e.g., Bradshaw et al., 2008; Juan-Jordá et al., 2022; Roff et al., 2018). This relatively recent timescale for diversity loss presents challenges for creating long-term baselines needed in conservation decision-making and human-wildlife conflict management, whereby historical patterns of species diversity, distribution (including habitat use), and community composition for many taxa are either understudied or unknown (e.g., Ferretti et al., 2008, 2018; Heithaus et al., 2007; McClenachan et al., 2016). This is particularly the case for century-to-millennia-scales of comparison (e.g., Burg Mayer & de Freitas, 2023).

Archaeology has much to contribute to deep-time baselines and elucidating the antiquity of human engagements with elasmobranch taxa through the study of zooarchaeological specimens. Robust zooarchaeological shark, and other elasmobranch, assemblages have been recovered from many archaeological sites across the world (the Americas [e.g., Prieto, 2023; Betts et al., 2012; Colvin, 2014; Rick et al., 2002; Gilson and Lessa, 2021; Lopes et al., 2016], the African continent and Arabian peninsula [e.g., Charpentier et al., 2020; Roberts et al., 2019], the Mediterranean [van Neer et al., 2005] across Oceania [Wright et al., 2016; Weisler and McNiven, 2016] and Asia [e.g., Boulanger, 2023; Langley et al., 2023; Boulanger et al., 2023, 2022, 2021, 2019; Kealy et al., 2020; O'Connor et al., 2019]). However, relative to bony fish specimens and other types of marine vertebrate taxa, zooarchaeological elasmobranch specimens are often difficult to identify beyond order, family, or genus based on morphology alone (Ono and Intoh, 2011), making it challenging to construct deep-time baselines with enough taxonomic resolution to support conservation needs. There are several reasons for this, but primarily it is because chondrichthyan skeletons are composed of hyaline cartilage, with some elements only partly calcified. As a result, it is the vertebral centra, teeth, spines, and dermal denticles that are mostly preserved in archaeological deposits, and although these are the most readily distinguishable elasmobranch parts, they are particularly difficult to identify to the species level due to uniformity and ubiquity among elements within and between many species (e.g., Rick et al., 2002). For example, centrum size, shape and number of vertebrae can be variable among individuals of the same species as well as between species (Springer & Garrick, 1964). Thus, ease of identification to species and quantifications of relative abundance (e.g., % number of identified specimens [NISP], % minimum number of individuals [MNI]) may not be straightforward between regions of the same vertebral column. Shark tooth morphology also differs interspecifically and depending on tooth position along the upper and lower jaws within species. Furthermore, it is important to note that challenges of specimen identification may also be compounded by lack of access to adequate (e.g., taxonomically and elementally representative) modern comparative skeletal collections for many shark species (Burg Mayer and de Freitas, 2023; Shepherd and Campbell, 2021). All these factors mean that it can be difficult to identify elasmobranch zooarchaeological specimens, and even more so within assemblages from world regions characterized by high elasmobranch taxonomic diversity within orders, families, and genera (e.g., *Carcharhinus* spp., *Sphyrna* spp.).

Motivated by a desire to better leverage zooarchaeological data within elasmobranch archaeology and historical ecological fisheries baselines, we present the results of an initial Zooarchaeology by Mass Spectrometry (ZooMS) analysis of elasmobranch specimens from a zooarchaeological assemblage at the Clupper site (8MO17). Clupper is an Ancestral Period[1] indigenous archaeological site located in the Florida Keys (Keys), USA (Figure 1); the Keys comprise a small-island archipelago renowned for its breadth of marine biodiversity, including several elasmobranch species (Table 1). The marine and estuary habitats of the Keys are essentially made up of "Bay" and "Ocean" waters, including shallower waters (e.g., < 2 m deep) protected by mangrove islands and deeper waters (e.g., 10-30 m deep) beyond embayments, respectively (Tinari and Hammerschlag, 2021).

The Keys can be characterized as a historical fishery with shifting baselines of fisheries health and persistent decline across a diversity of species throughout the 20th century (e.g., Ault et al., 2005; McClenachan, 2009), including sharks, rays, and sawfishes (Heithaus et al., 2007; Powers et al., 2013). Zooarchaeological assemblages from the Keys provide a record of deep-time fisheries, including centennial-to millennial-scale perspectives of taxonomic diversity, distribution, and human engagement (e.g., LeFebvre et al., 2022), but given the abovementioned limitations in the zooarchaeology of elasmobranch remains, the goals of this study were to: 1) investigate the efficacy of the collagen-fingerprint-based approach known as ZooMS (Zooarchaeology by Mass Spectrometry) in identifying zooarchaeological elasmobranch specimens to species; and 2) begin to build a species-level baseline of elasmobranch diversity present in the Keys approximately 700-1,000 years ago (Ardren et al., 2018) for the advancement of archaeological and historical ecological research.

We begin with background summaries of biomolecular methods in species identifications as well as elasmobranch skeletal structure to contextualize the ZooMS approach, followed by an overview of the Clupper site and current zooarchaeological analysis of elasmobranch specimens. Then we review the materials and methods of analysis with an emphasis on building a modern comparative baseline for collagen peptide mass fingerprints of this previously untried taxonomic group. The results of the analysis demonstrate the ability of ZooMS to achieve species-level identifications of zooarchaeological specimens and are presented within the context of contemporary elasmobranch diversity and habitats. Using the Clupper data as an exemplar, we highlight the implications of the results from archaeological perspectives of Ancestral Indigenous harvest, elasmobranch historical ecology, and future zooarchaeological research.

Background

Biomolecular methods of species identification

Although biomolecular methods offer an objective solution to the issue of species identification, they are largely underutilized for several reasons, including costs per sample, damage to specimens, and speed of interpretation in some cases. The analysis of ancient DNA is becoming increasingly more cost-effective (e.g., Seersholm et al., 2018), and will undoubtedly increase in use in the future, yet its greatest limitation is that of molecular preservation, whereby the few studies that have attempted to do so for shark taxa have focused on very recent material less than a few hundred years old (Ahonen and Stow, 2008; Nielsen et al., 2017) and even these can yield <50% success rates (e.g., Shepherd and Campbell, 2021).

Proteins on the other hand, particularly bone collagen, are thought to survive much longer, or at least better in warmer environments, such as those inhabited by most tropical shark species. Harnessing this greater longevity, a method of species identification by 'fingerprinting' collagen using mass spectrometry, referred to as 'ZooMS' (Zooarchaeology by Mass Spectrometry), was created over a decade and a half ago (Buckley et al., 2009; 2010), based initially on mammals for investigating animal husbandry practices (e.g., Buckley and Kansa, 2011; Price et al., 2013), with wider taxonomic use in studies on reptiles (Harvey et al., 2019; Guiry et al., 2024), birds (Eda et al., 2020), amphibians (Buckley and Cheylan, 2020), and fishes (Richter et al., 2011; Harvey et al., 2018; 2022; Rick et al., 2019; Guiry et al., 2020; Buckley et al., 2021; 2022) in comparison to the other 'lower vertebrate' groups.

Although there are more than 28 types of collagen known to exist within humans at some point in life (Ricard-Blum, 2011), 80-90% of the collagen in the body consists of types I, II and III, of which type I collagen is by far the most abundant. The triple helical structure common to all collagens is maintained through the presence of repeating amino acids, proline (Pro) and its modified form hydroxyproline (Hyp), which induce the twisting structure in each chain. Some collagen types are formed from three identical trains, with the cartilage-dominant type II collagen being a prime example. In contrast, the dominant type in bone, type 1 collagen, is made of genetically distinct chains, which in most vertebrates are made up of two identical chains (alpha 1 chains) and one genetically distinct (alpha 2) chain. In bony ray-finned fishes, type 1 collagen is composed of three distinct chains, where the third ($\alpha3(I)$) is a duplicate of the ($\alpha1(I)$) gene (Morvan-Dubois et al., 2003). However, this distinct third chain does not appear to exist in

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[1] Here, within Florida archaeology, the Ancestral Period refers to time spans prior to European colonization. The use of this term as a chronological descriptor of Indigenous history is supported and preferred by some U.S. federally recognized contemporary Indigenous Peoples, including the Seminole Tribe of Florida in south Florida (see https://stofthpo.com/seminole-history/).

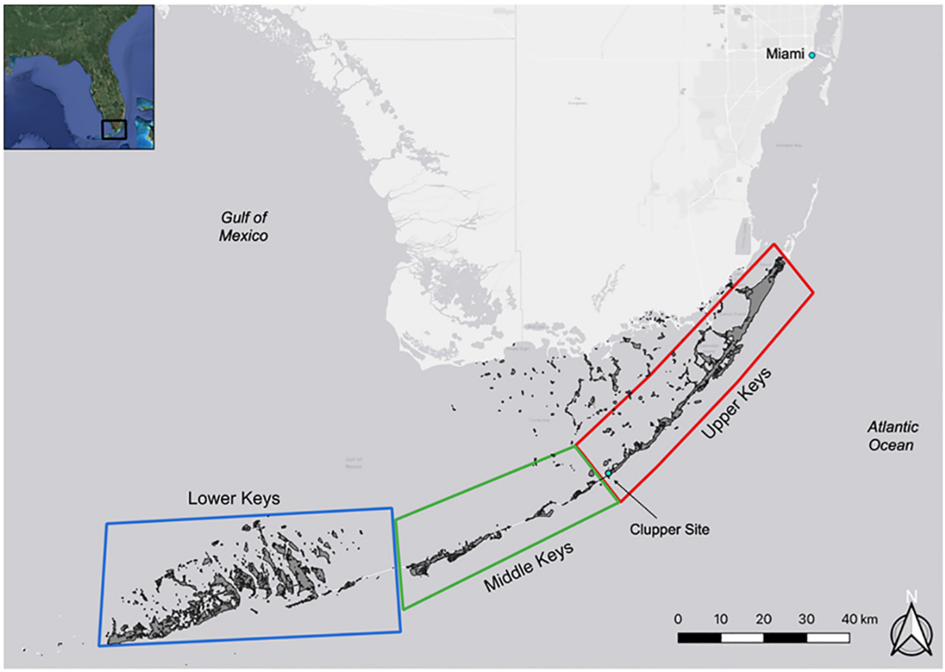

FIGURE 1
Location of the Clupper archaeological site (8MO17) within the Upper Keys, and distinctions between Upper, Middle, and Lower Keys.

sharks and lampreys (Kimura and Ohno, 1987), given the timing of the duplication event that gave rise to this (Harvey et al., 2021).

Elasmobranchs and their skeletal structure

The vertebrates are a subphylum (Vertebrata) within the phylum Chordata. The jawed vertebrates (gnathostomes) are a group comprising all tetrapods as well as the bony fishes (Osteichthyes), and the cartilaginous fishes (Chondrichthyes). During ontogeny, most vertebrate skeletons are initially composed mostly of hyaline cartilage that is largely replaced by bone via endochondral ossification (Hall, 1975); the biomineralization of skeletal tissues occurs through the deposition of biological apatite into collagen-rich (or amelogenin-rich, in the case of enamel and enameloid) matrices (Donoghue et al., 2006). However, as their name suggests, the skeletal structure of cartilaginous fishes remains primarily composed of cartilage, as they do not develop osseous skeletons, having secondarily lost this ability to produce endoskeletal bone (Coates et al., 2008). Elasmobranchs develop a relatively thin outer layer of cortical mineralization over most of their skeleton (Dean et al., 2015; Seidel et al., 2016), which is typically characterized by type II collagen, a triple helical molecule made up of three identical alpha chains (COL2A1).

The living chondrichthyans are split into two main groups, the Holocephali and the Elasmobranchii. The former are divided into three families, the Callorhincidae, Chimaeridae and the Rhinochimaeridae, whereas the latter are a much more diverse group, including the sharks (selachians; nine recognized orders) and the rays, skates, sawfishes and guitarfishes (batoids; four recognized orders). The cartilaginous elements (jaws, fins, and vertebrae) are covered by mineralized polygonal tiles called tesserae, more abundant in elasmobranchs, but also increasingly recognized in holocephalans (Pears et al., 2020; Seidel et al., 2020). It is the internal part of these tesserae, the round cells enclosed in lacunae, that contain the cartilaginous type II collagen (Seidel et al., 2017), whereas the external parts located on the perichondrial side are characterized by flatter cells that are engulfed in a type I collagen matrix (Orvig, 1951; Kemp and Westrin, 1979), known as fibrous perichondrium (Dean et al., 2015). In addition to this, as reviewed by Dean and Summers (2006), there are other types of mineralized tissues in the elasmobranch endoskeleton. Areolar mineralization characterizes the vertebral centra, and lamellar mineralization, comparable to bone tissue, has been recorded in the neural arches. The latter was first termed osseous tissue due to the presence of elongated cells like the osteoblasts of bone (Peignoux-Deville et al., 1982), expressing type I collagen genes (Enault et al., 2016), and because they are enclosed within a type I collagen-rich extracellular matrix that is able to mineralize (Eames et al., 2007); see Berio et al. (2021) for review. Therefore, although dominated by type II collagen, type I collagen typical of bone is estimated to account for about one third of the total collagen content of shark cartilage (Rama & Chandrakasan, 1984).

The Clupper site and elasmobranch specimens

Elasmobranch specimens and artifacts, especially from sharks (e.g., shark tooth drills), have been documented from coastal, island,

and inland Ancestral Period Indigenous archaeological sites across greater Florida, attesting to the cultural significance of such taxa in the past, including their use as food, tools, and items of personal adornment, ritual significance, and/or trade for several millennia (e.g., Early Archaic, ca. 6,000 BC [Farrell, 2021]; see also Keller and Thompson, 2013). The zooarchaeological specimens used in this study were selected from the Clupper site, an Indigenous village inhabited ca. AD 650-1,250 (Ardren et al., 2018) (Figure 1). The site is located on Upper Matecumbe Key, surrounded by or within proximity to a variety of maritime habitats, including sandy bottom flats, coral reefs, sea grass beds, rocky substrate, mangroves, marshes, and deeper waters. The site comprises a black earth midden. The midden is characterized by an abundance of well-preserved vertebrate fauna, invertebrate fauna, pottery and shell artifacts (Ardren et al., 2018; LeFebvre et al., 2022). Current zooarchaeological results from midden samples show that while bony fishes are the most abundant vertebrate taxon as represented per number of individual specimens (NISP = 9,350), followed by marine and freshwater turtles (NISP = 7,683), elasmobrachs are the third most relatively well-represented taxon with 578 NISP (Oliveira, 2024; see Supplementary Table S1). However, it is possible that elasmobranchs are underrepresented within the assemblage due to their cartilaginous structure, with representation being limited to vertebrae and teeth (LeFebvre et al., 2022; Rick et al., 2002).

The Clupper elasmobranch specimens were identified based on morphology using modern comparative skeletal specimens from the Environmental Archaeology Program (EAP) and South Florida Archaeology collections at the Florida Museum of Natural History, USA (LeFebvre et al., 2022). The taxonomic resolution achieved (Table 2) included specimens identified at the level of order (n = 2), family (n = 1), genus (n=3), species (n=4), and cf. (compares with) species (n=3). Order-level identifications to Carcharhiniformes (ground sharks; n = 229) and Rajiformes (flattened cartilaginous fishes; n = 101) accounted for the majority of individual specimen identifications. Carcharhinidae was the only family-level identification. The most abundantly identified shark taxon beyond order or family was the hammerhead genus (*Sphyrna* spp.). Spotted eagle ray (*Aetobatus narinari*) specimens were the most common Rajiformes identified beyond order. Other levels of genus, species, and cf. species identifications included nurse shark (*Ginglymostoma cirratum*), tiger shark (*Galeocerdo cuvier*), sandbar shark (cf. *Carcharhinus plumbeus*), lemon shark (cf. *Negaprion brevirostris*), Atlantic sharpnose shark (*Rhizoprionodon terraenovae*), sawfish (*Pristis*

TABLE 1 List of shark and ray species observed through contemporary survey efforts in south Florida (e.g., Miami and Florida Keys).

Family	Species	Common Name	Conservation status
Ginglymostomatidae	*Ginglymostoma cirratum*[+]	nurse shark	Vulnerable
Lamnidae	*Carcharodon carcharias*	white shark	Vulnerable
Carcharhinidae	*Carcharhinus acronotus*	blacknose shark	Endangered
Carcharhinidae	*Carcharhinus brevipinna*	spinner shark	Vulnerable
Carcharhinidae	*Carcharhinus leucas*	bull shark	Vulnerable
Carcharhinidae	*Carcharhinus limbatus*[+]	blacktip shark	Vulnerable
Carcharhinidae	*Carcharhinus obscurus*[+]	dusky shark	Endangered
Carcharhinidae	*Carcharhinus perezi*	Caribbean reef shark	Endangered
Carcharhinidae	*Carcharhinus plumbeus*	sandbar shark	Endangered
Carcharhinidae	*Negaprion brevirostris*[+]	lemon shark	Vulnerable
Carcharhinidae	*Rhizoprionodon terraenovae*[+]	Atlantic sharpnose shark	Least concern*
Galeocerdonidae	*Galeocerdo cuvier*[+]	tiger shark	Near threatened
Sphyrnidae	*Sphyrna lewini*[+]	scalloped hammerhead	Critically endangered
Sphyrnidae	*Sphyrna mokarran*	great hammerhead	Critically endangered
Sphyrnidae	*Sphyrna tiburo*[+]	bonnethead shark	Endangered
Pristidae	*Pristis pectinata*[+]	smalltooth sawfish	Critically endangered
Dasyatidae	*Hypanus americanus*	southern stingray	Near Threatened
Urotrygonidae	*Urobatis jamaicensis*	yellow round ray	Least concern*
Aetobatidae	*Aetobatus narinari*[+]	whitespotted eagle ray	Endangered

Data compiled from Heithaus et al. (2007); Tinari and Hammerschlag (2021), and Ramey (2021). Conservation status from IUCN (May 16, 2024). *IUCN population trends for all taxa are listed as "decreasing" except for Atlantic sharpnose shark as "increasing" and yellow round ray as "stable." [+] Species identified by ZooMS in the Clupper zooarchaeological elasmobranch assemblage.

TABLE 2 Elasmobranch taxonomic identifications based on morphology from the Clupper site, Upper Matecumbe Key, Florida, U.S.A. Levels of taxonomic identification span order to species.

Taxonomic ID	Level of ID	Common Name	NISP
Ginglymostoma cirratum	species	Nurse shark	25
Carcharhiniformes	order	Ground sharks	229
Carcharhinidae	family	Requiem sharks	40
cf. *Carcharhinus plumbeus*	cf. species	Sandbar shark	1
cf. *Negaprion brevirostris*	cf. species	Lemon shark	4
Rhizoprionodon terraenovae	species	Atlantic sharpnose shark	10
Galeocerdo cuvier	species	Tiger shark	2
Sphyrna sp.	genus	Hammerhead shark	124
Pristis sp.	genus	Sawfish	16
Rajiformes	order	flattened cartilaginous fish	101
Hypanus sp.	genus	Stingray	4
Hypanus cf. *sabinus*	cf. species	Atlantic stingray	7
Aetobatus narinari	species	Spotted eagle ray	15

spp.), stingray (*Hypanus* spp.), and Atlantic stingray (*Hypanus* cf. *sabinus*).

Interpretations of Indigenous fishing practices at Clupper indicate emphases on inshore habitats and a generalist, or multi-species approach, to fishing versus targeted or selective (LeFebvre et al., 2022; Oliveira, 2024). However, it is important to note that a generalist (i.e., multi-species) fishing strategy does not preclude targeted or preferential consumption of species caught. Rather, it refers to fisheries strategies that usually employ gear suited to capturing a diversity of species versus single or a selected few (Bieg et al., 2018). It is also the case that generalist fishing strategies are often not exclusively practiced and may include selective fishing approaches depending on species behaviors and availability (e.g., migrations, aggregations), as well as environmental conditions throughout a year (e.g., Ziegler et al., 2018).

The suggestion of a generalist strategy to fishing at Clupper is based on the identifications of bony fish and sea turtle taxa at genus or species levels and the overall high taxonomic diversity present in the zooarchaeological assemblage (LeFebvre et al., 2022). Moreover, genus- and species-level identifications indicate a fishery primarily focused on inshore habitats (Oliveira, 2024). Patterns of harvest over time have also been noted, including a decrease in almost exclusively marine taxa (e.g., sea turtles, grunts [*Haemulon* spp.]) concomitant with an increase in taxa that also frequent brackish waters, such as catfishes (e.g., *Ariopsis felis* and *Bagre marinus*) (Oliveira, 2024). Based on the schooling habits and daily migration patterns of the majority of bony fishes identified, fishing technology likely employed a mix of mass capture with nets and smaller catches via traps (Oliveira, 2024). Interpreting elasmobranch harvest has been less nuanced due to the lack of taxonomic resolution beyond order or family. Thus, shark, ray, and sawfish species harvest has been generally described as possible across essentially any habitat a shark or ray may inhabit (LeFebvre et al., 2022).

Materials and methods

Both modern and zooarchaeological elasmobranch specimens were sampled for this study. The selection and analysis of modern specimens was based on establishing a modern baseline of species identification, using ZooMS. Zooarchaeological specimens were selected to test the ability of ZooMS to confirm, refine, or refute previous identifications based on morphology.

Modern reference materials

Modern skeletal tissues (jaw and/or vertebrae; see Supplementary Table S2) were obtained for 39 species of 17 taxonomic families of elasmobranch, including all of those known to inhabit the oceanic and estuarine environments surrounding peninsular Florida and the Keys (Table 3). The specimens were selected from the modern skeletal comparative collection in the Environmental Archaeology Program at the Florida Museum of Natural History supplemented by specimens of two *Pristis* species sampled in triplicate from National Museums Scotland due to uncertainty in the taxonomic identification of the Florida Museum of Natural History (FLMNH) reference specimen. With a focus on sampling as much taxonomic diversity as possible relevant to the study region represented in the comparative collection, specimens included vertebrae, teeth, as well as dry-preserved cartilaginous materials.

Archaeological materials

Selection of zooarchaeological specimens from currently analyzed Clupper samples was aimed at either refining or testing the taxonomic breadth of specimen identifications made based on morphology (Supplementary Table S3). Specimens were analyzed from the following levels of identification: Order (Carcharhiniformes, Rajiformes), Family (Carcharhinidae), Genus (*Sphyrna* sp., *Hypanus* sp., *Pristis* sp.), species (*Aetobatus narinari*, *Rhizoprionodon terraenovae*, *Ginglymostoma cirratum*), and likely species (cf. *Hypanus sabinus*, cf. *Negaprion brevirostris*, cf. *Galeocerdo cuvier*). Analyzed specimens were from two 50 × 50 cm test pits excavated in 10 cm arbitrary levels. It is important to note that the focus of this initial effort was not quantitative in terms of identifying relative abundances of species represented at the site or trends in elasmobranch taxonomic diversity through time (i.e., across levels of excavation), but rather to gain as refined a taxonomic baseline as possible of species represented at the Clupper site during its Ancestral history from which to (re) consider continuing and future approaches to elasmobranch identification, quantification, and interpretation at Clupper and across the Keys more broadly.

TABLE 3 List of species included in this study (specimen details given in Supplementary Table S2).

Order	Family	Common Name	Scientific Name
Hexanchiformes	Hexanchidae	Bluntnose sixgill shark	*Hexanchus griseus*
Orectolobiformes	Ginglymostomatidae	Nurse shark	*Ginglymostoma cirratum**
Lamniformes	Carchariidae	Sand tiger shark	*Carcharias taurus*
Lamniformes	Lamnidae	Great white shark	*Carcharodon carcharias**
Lamniformes	Lamnidae	Shortfin mako shark	*Isurus oxyrinchus*
Lamniformes	Alopiidae	Common thresher shark	*Alopias vulpinus*
Carcharhiniformes	Triakidae	School shark/Tope shark	*Galeorhinus galeus**
Carcharhiniformes	Triakidae	Dusky smooth-hound shark	*Mustelus canis*
Carcharhiniformes	Hemigaleidae	Snaggletooth shark	*Hemipristis elongata**
Carcharhiniformes	Carcharhinidae	Blacknose shark	*Carcharhinus acronotus*
Carcharhiniformes	Carcharhinidae	Bignose shark	*Carcharhinus altimus*
Carcharhiniformes	Carcharhinidae	Spinner shark	*Carcharhinus brevipinna*
Carcharhiniformes	Carcharhinidae	Galapagos shark	*Carcharhinus galapagensis*
Carcharhiniformes	Carcharhinidae	Finetooth shark	*Carcharhinus isodon*
Carcharhiniformes	Carcharhinidae	Bull shark	*Carcharhinus leucas*
Carcharhiniformes	Carcharhinidae	Blacktip shark	*Carcharhinus limbatus**
Carcharhiniformes	Carcharhinidae	Dusky shark	*Carcharhinus obscurus*
Carcharhiniformes	Carcharhinidae	Caribbean reef shark	*Carcharhinus perezii*
Carcharhiniformes	Carcharhinidae	Sandbar shark	*Carcharhinus plumbeus*
Carcharhiniformes	Carcharhinidae	Night shark	*Carcharhinus signatus*
Carcharhiniformes	Carcharhinidae	Lemon shark	*Negaprion brevirostris**
Carcharhiniformes	Carcharhinidae	Blue shark	*Prionace glauca**
Carcharhiniformes	Carcharhinidae	Atlantic sharpnose shark	*Rhizoprionodon terraenovae*
Carcharhiniformes	Galeocerdonidae	Tiger shark	*Galeocerdo cuvier**
Carcharhiniformes	Sphyrnidae	Scalloped hammerhead	*Sphyrna lewini*
Carcharhiniformes	Sphyrnidae	Great hammerhead	*Sphyrna mokarran*
Carcharhiniformes	Sphyrnidae	Bonnethead	*Sphyrna tiburo*
Carcharhiniformes	Sphyrnidae	Smooth hammerhead	*Sphyrna zygaena*
Rhinopristiformes	Rhinobatidae	Atlantic guitarfish	*Pseudobatos lentiginosus*
Rhinopristiformes	Pristidae	Largetooth sawfish	*Pristis pristis*
Rhinopristiformes	Pristidae	Smalltooth sawfish	*Pristis pectinata*
Rajiformes	Rajidae	Clearnose skate	*Rostroraja eglanteria**
Myliobatiformes	Dasyatidae	Roughtail stingray	*Bathytoshia centroura*
Myliobatiformes	Dasyatidae	Southern stingray	*Hypanus americanus*
Myliobatiformes	Dasyatidae	Atlantic stingray	*Hypanus sabinus**
Myliobatiformes	Dasyatidae	Bluntnose stingray	*Hypanus say*
Myliobatiformes	Urotrygonidae	Yellow stingray	*Urobatis jamaicensis*
Myliobatiformes	Aetobatidae	Spotted eagle ray	*Aetobatus narinari*
Myliobatiformes	Rhinopteridae	Cownose ray	*Rhinoptera bonasus*

*Specimens studied for LC-MS/MS sequencing to improve marker identification.

ZooMS collagen peptide mass fingerprinting

Approximately 25-50 mg of modern and archaeological tissues were processed for collagen peptide mass fingerprinting following Buckley (2013), with modern tissues degreased beforehand by fully submerging twice in 83%/17% chloroform/methanol (first for 20 minutes, then for 3 hours). For collagen extraction, 1 mL 0.6 M hydrochloric acid (Sigma-Aldrich, UK) was added to each intact sample to enable decalcification. Half of the acid-soluble fraction was then ultrafiltered into 50 mM ammonium bicarbonate (Sigma-Aldrich, UK), with two centrifugation steps (20 min; 12,400 rpm) and recovered in 0.1 mL solution. This was then diluted 1/20 and 1 µL co-crystalized with 1 µL 10 mg/mL alpha-cyano hydroxycinnamic acid (Sigma-Aldrich, UK) in 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) onto a stainless-steel Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-ToF) mass spectrometric target plate. Using a Bruker Rapiflex MALDI-ToF instrument, up to 20,000 laser shots were acquired over the mass/charge (*m/z*) range 700-3,700 and resultant spectra of archaeological samples were manually categorized into 'groups' that each were composed of their own set of peptide markers. Peaks that appeared to differ between these groups were then compared with those present or absent in the associated reference material within this study, taking into consideration morphological identification for the majority in each archaeological group. Tandem mass spectra were also acquired via LC-MS/MS sequencing of selected modern specimens for improved results from database searching to assist confirmation of homologous markers between taxa.

LC-MS/MS sequencing

LC-MS/MS was carried out to improve understanding of the peaks observed in the ZooMS spectra (see Supplementary Table S3 for taxa selected to span range of biomarkers). Digested samples were analyzed using an UltiMate® 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to a QE HF (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile, and the column used was a 75 mm × 250 µm internal diameter 1.7 µM CSH C18, analytical column (Waters, UK). A 1 µl aliquot of the sample was transferred to a 5 µl loop and loaded on to the column at a flow of 300 nl/min for 5 minutes at 5% B. The loop was then taken out of line and the flow was reduced from 300 nl/min to 200nl/min in 0.5 minute. Peptides were separated using a gradient that went from 5% to 18% B in 34.5 minutes, then from 18% to 27% B in 8 minutes and finally from 27% B to 60% B in 1 minute. The column is washed at 60% B for 3 minutes before re-equilibration to 5% B in 1 minute. At 55 minutes the flow is increased to 300 nl/min until the end of the run at 60 minutes. Mass spectrometry data were acquired in a data-directed manner for 60 minutes in positive mode. Peptides were selected for fragmentation automatically by data-dependent analysis on a basis of the top 12 peptides with *m/z* between 300 to 1750 Th and a charge state of 2, 3 or 4 with a dynamic exclusion set at 15 seconds. The MS Resolution was set at 120,000 with an AGC target of 3 x10^6 and a maximum fill time set at 20 ms. The MS2 Resolution was set to 30,000, with an AGC target of 2 ×10^5, a maximum fill time of 45 ms, isolation window of 1.3 Th and a collision energy of 28. All data were collected in centroid mode. Raw files were then converted to mascot generic format (.MGF) files, which were searched against a locally curated database (Supplementary Table S4) of elasmobranch collagen type 1 (COL1A1 and COL1A2) and type 2 (COL2A1) sequences obtained from the protein BLAST search of the elephant shark (*Callorhinchus milii*). To assist evaluation of the manually selected peptide biomarkers, the resultant.MGF files for each Error Tolerant search were combined, filtered by removing peptides of <10 ion score, and sorted via *m/z* value.

Results

Peptide composition of the mass spectrometric fingerprints

The results indicate that, despite additional complexity of the collagen peptide mass fingerprint obtained due to the presence of the cartilaginous type II collagen, ZooMS provides exceptionally high taxonomic resolution in this group, yielding species-level identifications. Although only three of the species included in this study had known collagen type I (COL1A1 and COL1A2) and type II (COL2A1) sequences, *Hypanus sabinus, Pristis pectinata* and *Carcharodon carcharias* (see Supplementary Tables S5–S7 respectively for LC-MS/MS sequencing results), it was clear that a large proportion of peptides in the fingerprints derived from COL2A1, both in the archaeological (Figure 2) and the modern samples (Supplementary Figures S1, S2). Although differences in relative abundance of particular peaks could be observed in some of our peptide mass fingerprint comparisons between each sampled modern tissue type (e.g., tooth vs. vertebrae; Supplementary Figures S3–S5), they were not unexpected, reflecting the difference in dominant protein, whether COL1 or COL2.

Taxonomic resolution: modern comparative baseline

Species-level differences between the collagen peptide mass fingerprints for modern comparative specimens could readily be observed (Supplementary Figures S6–S16), even for each one of the 12 species of *Carcharhinus* and the four species of *Sphyrna* analyzed here (Table 3). Sampling of the two *Pristis* species of relevance to this study showed that the initially supplied reference material from *P. pristis* was incorrect, deriving from *P. pectinata*, more in keeping with geographical origins of the reference material. These differences, proposed as species biomarkers (Supplementary Table S8; Supplementary Figures S17–S51), were clearly visible in the

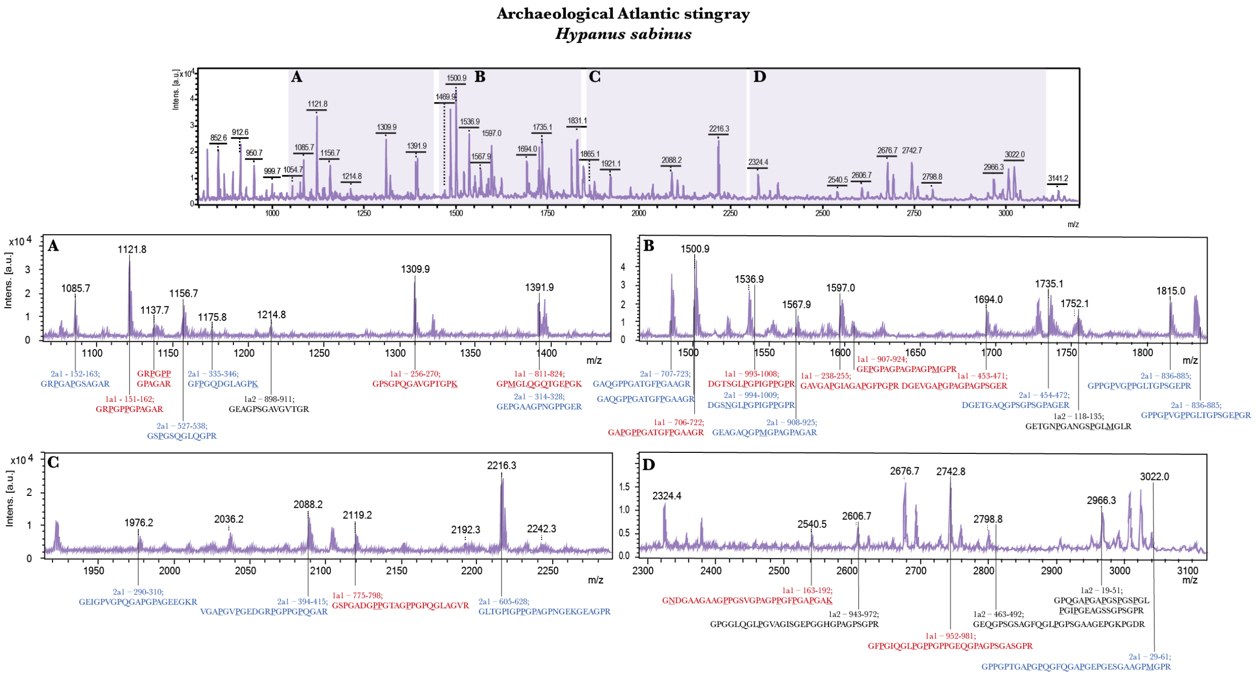

FIGURE 2

Sequence-annotated MALDI peptide mass fingerprint (top spectrum) of dominant proteins extracted from a confidently identified archaeological *Hypanus sabinus* vertebra (black text = COL1A1, red text = COL1A2, blue text = COL2A1; matched to peptides listed in Supplementary Table S5) with zoomed in sections **(A–D)** in order of increasing *m/z*.

peptide mass fingerprints of the archaeological specimens also (Figure 3).

Taxonomic resolution: zooarchaeological baseline

Although just over 30% (n=41) of the 137 analyzed archaeological samples did not yield suitable quality collagen fingerprints, the 98 specimens that did covered a greater than expected range of taxonomic diversity based on morphological identification, with 12 distinct groups identified (Figure 4). While the majority of these results derived from specimens that could not be morphologically identified below the family level (i.e., Carcharhinidae) and often order level, 48 of our samples did derive from a genus-level or lower suggested identification, though mostly (n=33) from the hammerheads *Sphyrna* spp. (Supplementary Table S3); of the 40 of these that were successful (7 of the 8 poor collagen samples were of *Sphyrna*, with one from *Galeocerdo*), 10 were initially incorrect on the original morphological identification, and one of these (#78) cautioned/corrected prior to ZooMS analysis (initially considered *Rhizoprionodon terraenovae* and later suggested on morphological grounds as potentially deriving from hammerhead [*Sphyrna*]), an identification confirmed by ZooMS and improved to the level of the bonnethead (*S. tiburo*). All other misidentifications were from specimens morphologically identified as hammerhead, corrected to Atlantic sharpnose shark (*R. terraenovae*) in six instances (#70, 87, 99, 101, 103, 110), the lemon shark (*Negaprion brevirostris*) in two instances (#66, 95), and the blacktip shark (*C. limbatus*) in one instance (#61). These results are suggestive of misleading morphological criteria used between the two taxonomic groups during initial identification. However, we also note the value in morphological identification, which remains important given the substantial number of specimens that do not yield collagen/biomolecular information, which, in this case comprised as much as approximately one third of the samples analyzed in this study, and is likely to have taphonomic biases that relate to species and element.

Consistent with the morphological identifications, the most abundant shark taxon observed via ZooMS among the Clupper specimens was Carcharhinidae (i.e., migratory live-bearing sharks of warm seas). Within this family, the lemon shark was the most frequently identified among the sampled specimens. There are only two species of lemon shark within the genus *Negaprion*. The lemon shark (*N. brevirostris*) inhabits oceanic waters of the Americas, and the sicklefin lemon shark (*N. acutidens*) inhabits the Indo-Pacific. Lemon sharks prefer shallow coastal waters, often feeding at night (Wetherbee et al., 1990) and growing to >3 m in length (Dibattista et al., 2007). Lemon sharks are abundant in the Keys and are known to inhabit inshore marine habitats, such as coral reefs or flats, as well as estuaries (Compagno, 1984; Jennings et al., 2008; Tinari and Hammerschlag, 2021; Wiley and Simpfendorfer, 2007).

Carcharhinus is the largest genus of the Carcharhinidae family, containing at least 35 different species. Of those identified at Clupper, the finetooth shark (*C. isodon*) is commonly found in the western Atlantic from North Carolina to Brazil. Relatively small (1.6-1.7 m) and fast swimming, it forms large schools in shallow inshore coastal

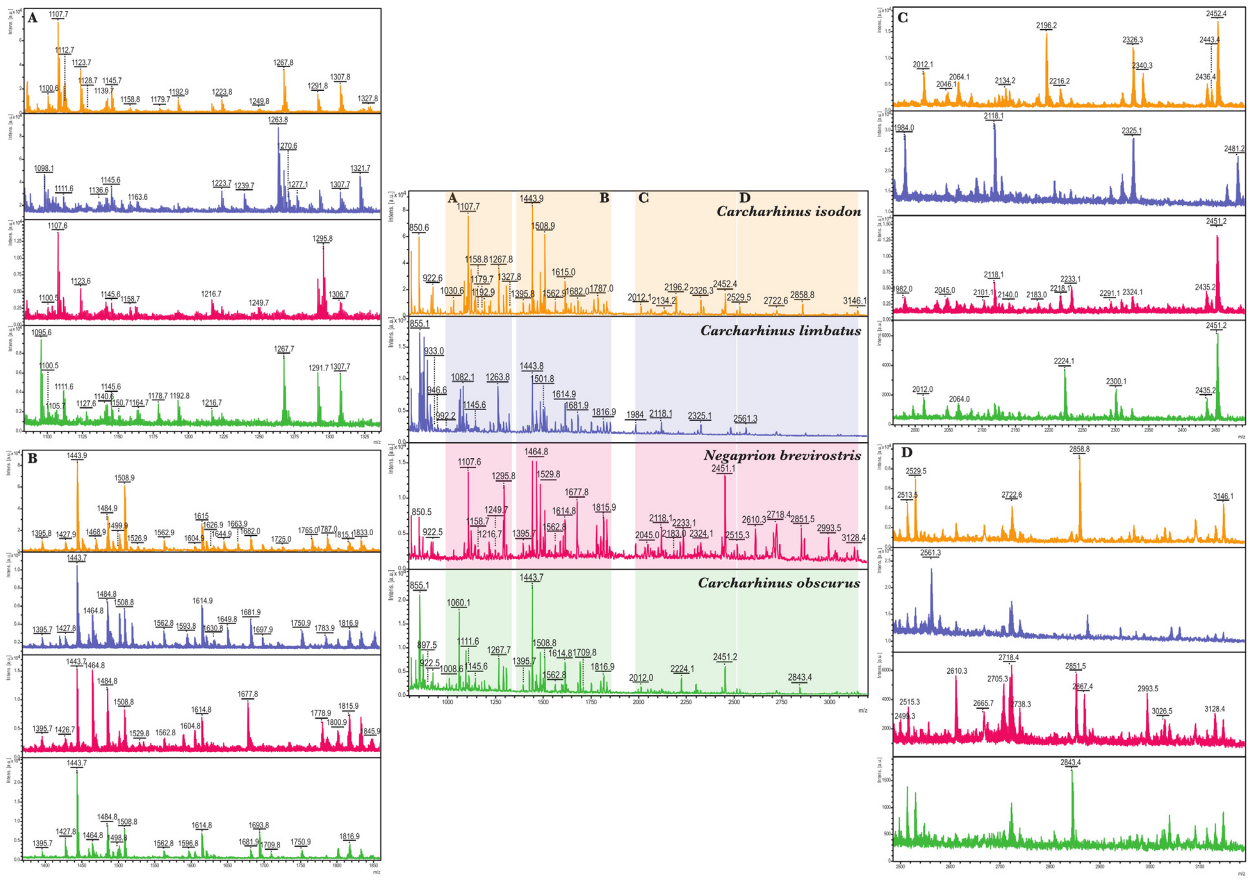

FIGURE 3
Example peptide mass fingerprints showing taxonomic resolution amongst archaeological samples of carcharhinids; central portion shows the most diagnostic regions **(A–D)** for species determination of the mass spectra for each of the four elasmobranches *Carcharinus isodon* (orange; top), *C. limbatus* (blue; second to top), *Negaprion brevirostris* (red; second to bottom) and *C. obscurus* (green; bottom) surrounded by zoomed in spectra **(A–D)** of increasing *m/z*.

waters and habitats (Compagno, 1984). It is known to migrate seasonally to follow warm waters (Castro, 1993), including those surrounding Florida during the winter. Also identified within the Clupper assemblage, the blacktip shark (*C. limbatus*) has a worldwide distribution in tropical waters and is usually found in groups of varying size in waters less than 30 m deep, including mangrove and estuary habitats, as well as favoring island lagoons and drop-offs near coral reefs (Castro, 1996). The blacktip is described as a stocky species, typically reaching between 1.2 to 1.9 m, and only rarely reaching 2 m in length (Castro, 1996; Compagno, 1984). While this species is present in waters of the Gulf of Mexico, southern Florida and the Keys year-round (Tinari and Hammerschlag, 2021; Wiley and Simpfendorfer, 2007), it tends to be most abundant during winter months after migrating southward from nursery grounds along the northern Gulf coast, Carolinas, and Georgia (Castro, 1996; Heithaus et al., 2007). It occurs in inshore habitats with shallower waters. The dusky shark (*C. obscurus*) is one of the largest members of this genus, at 3-4 m in adult length; it spends most of its time at 10-80 m depths and within the western Atlantic migrates south during the winter (Natanson and Kohler, 1996).

The Atlantic sharpnose shark (*R. terraenovae*), the third most identified shark in this study, is also a relatively small shark (~1 m adult length) (Compagno, 1984). It is one of the most frequently encountered sharks in subtropical waters off the north-western Atlantic Ocean today, including Florida, and the Gulf of Mexico (Branstetter, 1990; Marquez-Farias and Castillo-Geniz, 1998; Parsons and Hoffmayer, 2005). It is considered a coastal species that often engages in regular inshore and offshore migration to depths of up to 280 m (Compagno, 1984). It is found in a variety of habitats such as shallow seagrass beds to deep non-vegetated sand or mud and utilizes a series of coastal bays and estuaries as nurseries for juvenile development (Carlson et al., 2008). Atlantic sharpnose sharks are year-round residents of the Gulf of Mexico, Florida and the Keys, but are most common during the winter (Tinari and Hammerschlag, 2021); they are similar in length to bonnetheads, with the morphology of the vertebrae being especially difficult to distinguish between the two species.

The tiger shark (*Galeocerdo cuvier*) is the only extant species in the family Galeocerdonidae. It is a large (>5 m), solitary, mostly nocturnal hunter, moving closer to shore to feed (Compagno, 1984). It inhabits coastal and open ocean environments, and is highly mobile, engaging in ontogenetic and seasonal migrations (Ajemian et al., 2020; Lea et al., 2015). It prefers tropical waters during the winter (e.g., Caribbean and Florida) and moves to high-

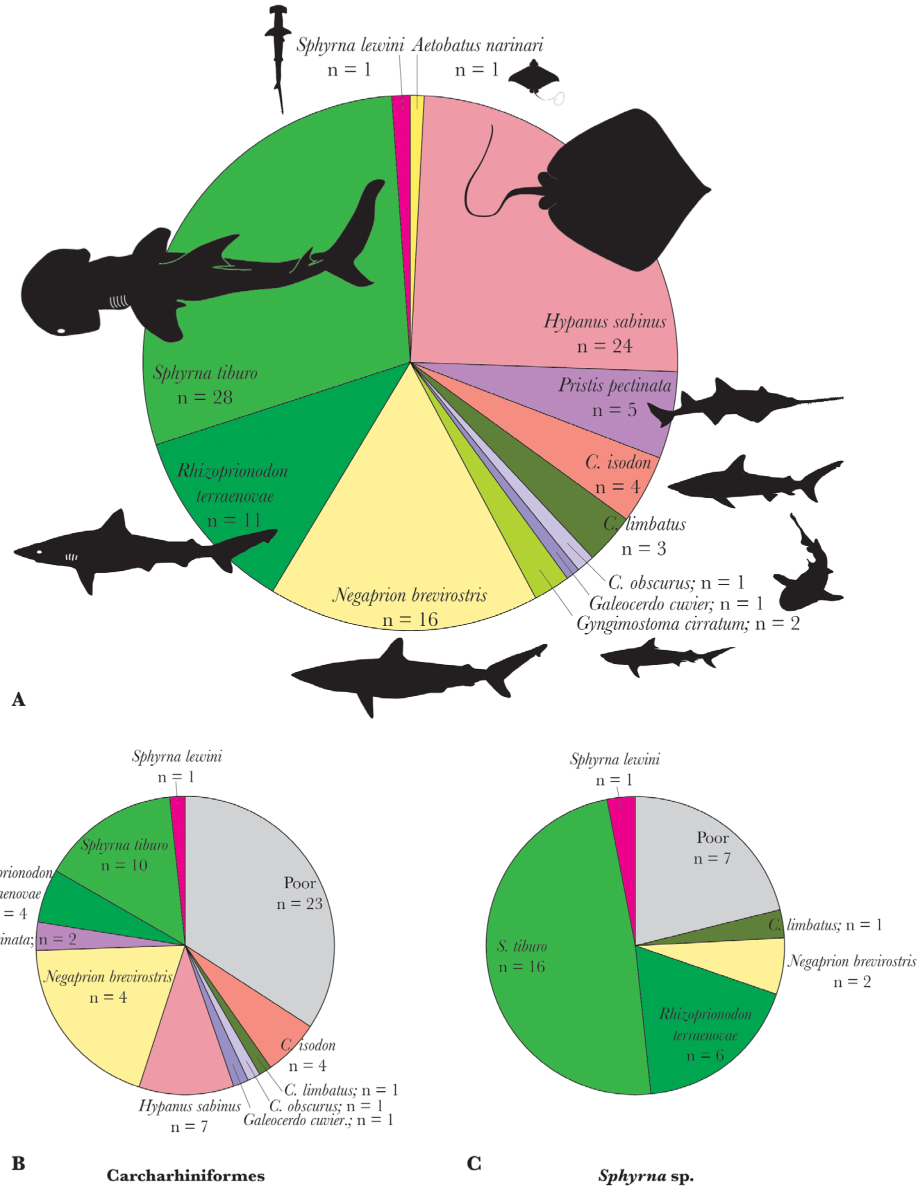

FIGURE 4

Pie charts showing **(A)** species composition of archaeological remains analyzed by ZooMS, **(B)** the ZooMS results of species identified morphologically as Carchariniformes, and **(C)** the ZooMS results of species identified morphologically as *Sphyrna* (bottom right).

latitude oceanic areas in the summer (Lea et al., 2015). Although it is known to inhabit a variety of inshore habitats, such as estuaries, reefs, and lagoons (Compagno, 1984), there is also an increased use of continental-slope and deep-water habitats with increasing size (Ajemian et al., 2020). Off the coast of Florida, it is most often found in deeper oceanic waters (Tinari and Hammerschlag, 2021). The nurse shark (*Ginglymostoma cirratum*) is the only species of shark from a different order than ground sharks, the Orectolobiformes. It is a very common inshore, bottom-dwelling shark, often inhabiting waters of one meter or less deep and up to 12 m. It is typically ~3 m in size and is found on rocky reefs, channels between mangrove keys, and sand flats (Compagno, 1984). It is the most common shark found year-round in the shallow waters of the Gulf of Mexico, Florida, and the Keys (Heithaus et al., 2007; Tinari and Hammerschlag, 2021; Wiley and Simpfendorfer, 2007). Nurse sharks are largely nocturnal, often described as sluggish during the daytime, and frequently found aggregating in large sedentary resting groups (Compagno, 1984).

After the requiem sharks mentioned above, the second most frequently identified shark taxonomic group was the hammerheads, of the genus *Sphyrna*. Hammerheads are common, coastal inshore sharks among the Keys, and like most sharks, they are solitary hunters in the evening. The hammerhead species identified from Clupper were the bonnethead (*S. tiburo*) and scalloped hammerhead (*S. lewini*). The bonnethead is the smallest of hammerhead species, typically <1 m in length, but can reach up to 1.3 m in size (Carlson and Parsons, 1997; Compagno, 1984). This species prefers inshore, coastal habitats such as seagrass beds, shallow bays, and estuaries primarily ranging from 10-25 m in depth (Compagno, 1984; Heupel et al., 2006). In fact, this species tends to be a long-term resident of these environments (e.g., Pine Island Sound, Florida Bay, and Lower Florida Keys) with some residents not undertaking long coastal migrations (Heithaus et al., 2007; Heupel et al., 2006; Wiley and Simpfendorfer, 2007). Although bonnetheads do not engage in true schooling, they are known to aggregate together frequently (Compagno, 1984). The scalloped hammerhead is a large hammerhead species, reaching a maximum size of about 3.7 to 4 m in length (Compagno, 1984). It frequents coastal warm temperate and tropical seas, alternating between coastal and pelagic phases. It can be found over continental and insular shelves (<200 m depth) and in adjacent deep waters (Gallagher et al., 2014; Wells et al., 2018). The scalloped hammerhead also frequents shallow bays and estuaries, with nurseries typically located inshore (Corgos & Rosende-Pereiro, 2022). It is considered partly migratory and highly mobile with natural reefs and hard-bottom outcroppings being important foraging areas (Compagno, 1984; Wells et al., 2018). Scalloped hammerheads are the only species of large-bodied shark that engages in highly organized complex social schooling behavior (Gallagher et al., 2014; Klimley, 1985; Klimley and Nelson, 1981).

With regards to the batoids, three species were identified in this study, with a possible unconfirmed fourth (#18). There were at least two rays of the order Myliobatiformes, including the Atlantic stingray (*Hypanus sabinus*) and the spotted eagle ray (*Aetobatus narinari*), which is the only species of its genus found in the Atlantic (Richards et al., 2009). They can both be found regularly in the western Atlantic Ocean, including the Keys, but with different habitat preferences. The Atlantic stingray can reside in low salinity and has a high tolerance for shallow estuarine and freshwater habitats; spotted eagle rays are known to occur in inshore marine waters, including reef and mangrove habitats, but the species largely prefers open waters (DeGroot et al., 2021).

The smalltooth sawfish (*Pristis pectinata*) was the third identified batoid of the order Rhinopristiformes from among the analyzed specimens. While once found on both sides of the Atlantic Ocean (e.g., Bigelow and Schroeder, 1953), this species is now restricted to waters surrounding Florida, particularly within the Everglades National Park, and the Bahamas (Carlson et al., 2013; Simpfendorfer and Wiley, 2005; Wiley and Simpfendorfer, 2007). The species is present all year in south Florida coastal waters, and individual animal size tends to correlate with habitat preferences; with small individuals or juveniles observed in shallow coastal habitats, such as mangroves and estuaries, while larger juveniles or adults are observed among deeper or open waters (Waters et al., 2014).

Discussion

Mitigating limitations in identification

Given the challenges of elasmobranch identification in zooarchaeology (e.g., Gilson and Lessa, 2021; Kozuch and Fitzgerald, 1989; Prieto, 2023), and especially within tropical or subtropical environments with high biodiversity such as the Keys, the results of our analysis with the Clupper specimens are an exemplar of the methodological promise that ZooMS holds for improving elasmobranch species identifications across several world regions and taxa. From the perspective of taxonomic diversity and overcoming limitations in zooarchaeological identification via ZooMS, there are now at least nine different confirmed (including corrected identifications) shark species, two stingray species, and one sawfish species documented for the site. The high species-level taxonomic resolution achieved, for the first time, has implications for advancing archaeological and historical ecology research in the Keys and elsewhere.

Implications for Ancestral Indigenous harvest and elasmobranch historical ecology in the Keys

When considered within the context of contemporary elasmobranch diversity and distribution, the results provide a new opportunity to think beyond generalities gleaned from order- or family-level identifications and information (e.g., LeFebvre et al., 2022; Oliveira, 2024). Here we focus on contemporary species occurrence and co-occurrence observations from the Keys as a foundation for deeper interrogation of elasmobranch harvest and historical ecology represented by the Clupper data.

As part of a recent study investigating shark assemblages spanning state and federal management areas from Miami into the middle Keys (including Upper Matecumbe Key), Tinari and Hammerschlag (2021) (see also Table 1) assessed relationships between species occurrences and abundances by habitat type (i.e., Bay or Ocean), depth, and season (i.e., wet season from May-October and dry season from November-April). They also considered correlations between species size and occurrence. The authors found that while shark assemblages within the study region are characterized by year-round occurrences for the majority of observed species [see Table 1 in Tinari and Hammerschlag (2021)], there are also species-level nuances to occurrences. For example, nurse, tiger, and Atlantic sharpnose sharks have higher probabilities of occurrence in offshore habitats (i.e., ocean), compared to blacktip and lemon sharks found in inshore habitats (i.e., bay). Also, while temperature is not a significant driver of shark occurrence, salinity is for two of the most common species in the Keys, i.e., nurse sharks (i.e., decrease in occurrences with increases in salinity) and lemon sharks (i.e., increase in occurrences with increases in salinity). Depth is also a factor; tiger sharks occur in deeper waters, and nurse, blacktip, and lemon are found in shallower waters. Finally, there are variable correlations between some species sizes (i.e., total length) and season and habitat; whereby, occurrences of larger nurse and bull sharks are higher in the Keys, usually in offshore waters, during the dry season compared to Miami, larger lemon sharks have relatively higher occurrence in offshore habitats overall, and larger Atlantic sharpnose sharks occur more frequently in the Keys compared to waters near Miami.

Like species occurrences, fishery species co-occurrences and community composition are usually related to overlapping habitat preferences and dietary habits, both of which may vary throughout a day (e.g., nocturnal versus diurnal feeding) and/or season, as well as across sex, age, and reproductive cycles of different species (Heithaus et al., 2007). For the Keys and most germane to this study, nurse and bonnethead sharks are among the more frequently observed sharks in the Keys today and they have high rates of co-occurrence with several other elasmobranch species, including an inshore habitat overlap with sharpnose and lemon sharks, as well as with the southern and white-spotted eagle rays (Ramey, 2021).

Assuming contemporary patterns in elasmobranch species occurrence and co-occurrence, as well as the current environmental conditions of the Keys and surrounding waters, extend into the deep-past, our species-level results from the Clupper site suggest that several of the sharks and rays represented would have been opportunistically, and/or predictably, available for harvest from the same habitats as part of a generalist, primarily inshore, fishery at Clupper (e.g., LeFebvre et al., 2022; Oliveira, 2024). Common among inshore, shallow water (e.g., Bay) habitats, we can hypothesize that species, such as lemon, nurse, blacktip, and bonnethead sharks, were the most commonly harvested species based on their likely proximity to the shorelines of Upper Matecumbe Key and that their smaller overall body size was amenable to net and trap capture. We can also hypothesize that co-occurrences between elasmobranch and bony fishes also likely shaped the fishery given that the most frequently identified bony fish taxa from Clupper include catfishes (*Ariopsis felis* and *Bagre marinus*), grunts (*Haemulon* spp.), jacks (*Caranx* spp.), snappers (*Lutjanus* spp.), and groupers (*Epinephelus* spp. and *Mycteroperca* spp.) - all of which are commonly encountered among the same inshore habitats as the identified elasmobranch species. Species dietary patterns also have implications for hypotheses regarding elasmobranch harvest. For example, scalloped hammerhead, tiger, and lemon sharks are known to feed on bony fishes (e.g., jacks, catfishes), sea turtles, rays, and/or relatively smaller sharks (e.g., blacktip shark, nurse shark) within inshore habitats (e.g., Compagno, 1984), all of which are represented within the Clupper zooarchaeological record.

However, these hypotheses, largely based on co-occurrence inferences, do not necessarily preclude possible offshore or deeper-water harvest practices at Clupper that aimed to target more pelagic species. Three of the largest-growing elasmobranch species identified among the study specimens, the dusky shark, scalloped hammerhead, and white-spotted eagle ray, are also known to inhabit deeper, offshore waters as adults, depending on the time of day, seasonal migrations, and reproduction habits. This is similar to the largest-growing bony fish identified from Clupper, the sailfish (*Istiophorus platypterus*) (Oliveira, 2024). These taxa may have been harvested while crossing between offshore (i.e., ocean) and inshore (i.e., bay) habitats of the Keys. The Clupper site is located adjacent to a natural deep-water pass that would have made such crossings possible.

We can also begin to think more critically about technological approaches to elasmobranch harvest at Clupper, all of which would have been informed by traditional ecological knowledge developed across generations of cultural practices at the village and more broadly across the Keys (Oliveira, 2024). Approaches likely included several types of gear, including multi-species harvest techniques, such as nets and/or traps intended to capture a diversity of species at once (e.g., elasmobranchs and bony fishes), or those intended for one individual at a time, such as single hook and line and/or spear (Walker 2000). For example, contemporary commercial and recreational fisheries data show that finetooth sharks may be efficiently harvested using gillnets (Portnoy et al., 2016), while blacktip, dusky, and bonnethead sharks are captured using baited hook and line (e.g., individual angling or longline) (e.g., Ulrich et al., 2007). Species such as nurse sharks are also known to take bait intended for other fishes and raid fish nets or traps (Castro, 2000). Testing these ideas and hypotheses will require larger sample sizes identified to species for the calculation of relative abundances, as well as measurements of individual sizes and approximate ages represented across taxa.

From a geographic perspective of contemporary species occurrences, all the elasmobranch species identified from Clupper, except for finetooth shark, are known to inhabit the Keys today. The finetooth shark is not regarded as common in the Keys based on contemporary survey data of species distribution. While the finetooth shark has long been known to inhabit the waters off the peninsular coast of Florida during the fall and into winter (Castro, 1993), there was in 2007 the report of a new southern extent of the species occurrence to include Florida Bay waters along the southern terminus of peninsular Florida (Wiley and Simpfendorfer, 2007), well beyond the previously documented southern extent of the species around Lemon Bay on the southwest coast and Port Salerno on southeast coast of the peninsula; the authors reported that the presence of finetooth sharks in Florida Bay was likely rare overall, but significant from the perspective of possible exchange between Gulf and Atlantic stocks and learning more about the species' seasonal migration movements and relationship to water temperature. The species identification of finetooth shark via ZooMS at Clupper may have several implications from historical ecological and archaeological perspectives, including: 1) finetooth sharks were more common in the island region and/or greater Florida Bay in the past; 2) there is a potentially deeper history for stock exchanges through time; and 3) a finetooth shark, or portion of an individual, was transported to the Clupper site post mortem from a more northern location along the Florida peninsula. It could also indicate sea temperatures in the past implying environmental/climatic change. To test these possibilities, it will be imperative to identify more finetooth shark specimens within the Clupper zooarchaeological assemblage, from additional zooarchaeological assemblages from the greater Keys region, and ideally from across southern portions of peninsular Florida that are dated to the Ancestral Period.

In summary, we now have a more taxonomically refined and accurate understanding of the elasmobranch diversity represented at Clupper than we did prior to the ZooMS analysis, presenting an opportunity to think beyond generalities gleaned from primarily order- or family-level identifications and information. The data indicate that inshore, and likely to a lesser extent offshore, habitats local to or near the site supported a taxonomically diverse elasmobranch fishery ca. AD 1,000-1,300. It is reasonable to assert that elasmobranch harvest may have been linked to the harvest of other taxa (e.g., bony fishes and sea turtles) and facilitated using multi-species capture nets and/or traps. This may have been most prevalent among smaller species, such as finetooth sharks, blacktip sharks, and bonnetheads in shallower-water habitats. Multi-species capture methods may have also provided opportunities for opportunistic spear fishing of elasmobranchs attracted to fishes caught in nets or traps, such as nurse or bull sharks. Concomitant with inshore harvest and variable uses of fishery technology, some of the species represented may have also been pursued through more targeted approaches, such as hook and line, following seasonal availability depending on aggregation (e.g., *S. lewini* and *S. tiburo*) and/or migration habits across both inshore and offshore waters, including larger, migrating species more abundant within the Keys during the winter months (e.g., hammerhead and tiger sharks).

Implications for future zooarchaeological research and supporting conservation

The application of ZooMS to the study of elasmobranchs has potential for improved historical ecological baselines derived from zooarchaeological assemblages, and particularly in support of conservation. Because marine resource managers, conservation practitioners, and fisheries scientists often work with species-level data, the results offer an opportunity to consider how to better leverage zooarchaeological analyses and data to support species-specific as well as community-level historical baselines of elasmobranch diversity and distribution in the Keys (e.g., Tinari and Hammerschlag, 2021), albeit through a cultural lens of human selection and post-depositional taphonomic processes (e.g., preservation). Nonetheless, the species identified among the Clupper specimens are now known to have a deep-time history of human engagement and harvest, pre-dating 20th and 21st centuries scales of impact. Approaching the Clupper zooarchaeological species list as a historical survey, similar to more recent survey lists (e.g., Ramey, 2021; Tinari and Hammerschlag, 2021; see also Table 2), but providing a baseline of species occurrences well before the precipitous losses of recent history, we suggest three lines of possible future research, aimed at elucidating trends (e.g., stasis or change) in species diversity through time, across space, and among groups of people (i.e., cultural practices).

1. Given that almost all identified species from Clupper are extant, we can target specific species for genetic analyses leveraging aDNA (e.g., Shepherd and Campbell, 2021), and ultimately having the potential to contribute to critically needed long-term phylogenetic perspectives of elasmobranch species diversity and loss through time. The one as yet unidentified ray leaves open the potential for new species discovery, at least likely locally extinct.
2. With the use of high-throughput digital scanning techniques linked with ZooMS (e.g., Buckley et al., 2021), we can compare fragmented, species-identified elasmobranch zooarchaeological specimens with modern comparative specimens for the possible identification of species-specific morphological hallmarks not previously recognized.
3. Because elasmobranch species occurrences can be highly variable in terms of individual age and size within a given habitat, depending on many factors (e.g., nursery locations, water depth, salinity, adult migration patterns), and particularly within habitat-diverse tropical locations such as the Keys, we can begin to leverage ZooMS-identified specimens, including bulk-assemblage approaches (e.g., Buckley et al., 2016; Oldfield et al., 2024), to link specimen identification with allometric size data across species (e.g., species length and age correlations). These data would allow us to measure species sizes (and approximate ages), represented within the zooarchaeological record, and quantify relative species abundances accordingly (e.g., MNI, NISP), ultimately contributing to deep-time perspectives of species sizes beyond more recent time scales (e.g., decadal) as well as more specific interpretations of elasmobranch harvest habitats and practices in the past.

Not surprisingly, difficulties surrounding species-level elasmobranch identification of zooarchaeological specimens can significantly limit the information that can be garnered from preserved bone, teeth, and denticles, including insights into possible habitats of harvest and capture methods used in the past. Furthermore, identifications only to order or family level seriously diminish the historical ecological implications that can be drawn, which often necessitate species-level resolution and inference to support conservation and understanding the impacts of human predation through time (e.g., harvest for subsistence, shark fin markets). Archaeological elasmobranch assemblages, such as Clupper, are composed of specimens that hold critical potential to constructing as taxonomically, geographically, and temporally as possible baselines of diversity through time, and particularly within the context of long-term human engagement. The species identified from Clupper include several taxa considered to be vulnerable, endangered, or critically endangered (Table 1), particularly those characterized by K-selected traits, making them incredibly vulnerable to loss as noted earlier. While the use of ZooMS in establishing robust species-level identifications of elasmobranchs has been successful, its future use will allow us to answer many more questions and develop methodological advances that are not possible based on morphological identifications alone, perhaps most suitably to applications involving wildlife forensics, including the population-decimating shark fin trade (Cardeñosa et al., 2022). However, we assert that both ZooMS and morphology are integral to realizing the full potential of zooarchaeological data to contribute to conservation baselines and deep-time historical perspectives in the Keys and beyond.

Data availability statement

The original contributions presented in the study are available on FigShare at https://figshare.com/articles/dataset/Clupper_elasmobranch_species_identification_using_ZooMS/27087190?

file=49358374 and presented as additional figures in the Supplementary Material, further inquiries can be directed to the corresponding author/s.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because deceased animal tissues from museum collections were used.

Author contributions

MB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. E-MO: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. CO: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft. CB: Data curation, Formal analysis, Visualization, Writing – original draft. AK: Resources, Writing – original draft. NF: Resources, Writing – review & editing. TA: Resources, Writing – review & editing. VT: Writing – review & editing. SF: Writing – review & editing. ML: Resources, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1500595/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Sequence-annotated MALDI peptide mass fingerprint (top) of dominant proteins extracted from modern *Hypanus sabinus* tissue (black text = COL1A1, red text = COL1A2, blue text = COL2A1; matched to peptides listed in Supplementary Table S5) with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 2
Sequence-annotated MALDI peptide mass fingerprint (top) of dominant proteins extracted from modern *Carcharodon carcharias* tissue (black text = COL1A1, red text = COL1A2, blue text = COL2A1; matched to peptides listed in Supplementary Table S5) with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 3
MALDI spectra showing peptide mass fingerprints (top) of different modern tissues (vertebral process cartilage and tooth) from modern *Carcharhinus limbatus*, with zoomed in sections **(A-C)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 4
MALDI spectra showing peptide mass fingerprints (center) of different modern tissues (cranial cartilage and vertebra) from *Ginglymostoma cirratum*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 5
MALDI spectra showing peptide mass fingerprints (centre) of different modern tissues (jaw, tooth and 'cranial cartilage') from *Galeocerdo cuvier*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 6
MALDI spectra showing peptide mass fingerprints (top) of modern *Carcharhinus isodon, Carcharhinus limbatus, Carcharhinus signatus* and *Carcharhinus leucas*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 7

MALDI spectra showing peptide mass fingerprints (top) of modern *Carcharhinus galapagensis, Carcharhinus brevipinna, Carcharhinus obscurus* and *Carcharhinus altimus*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 8

MALDI spectra showing peptide mass fingerprints (top) of modern *Carcharhinus perezi, Carcharhinus plumbeus*, and *Carcharhinus acronotus*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 9

MALDI spectra showing peptide mass fingerprints (top) of modern *Sphryna lewini, Sphryna mokarran, Sphryna tiburo* and *Sphryna zygaena*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 10

MALDI spectra showing peptide mass fingerprints (centre) of modern *Galeorhinus galeus, Carcharias taurus, Pristis pristis* and *Pristis pectinata*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 11

MALDI spectra showing peptide mass fingerprints (top) of modern *Rhizoprionodon terraenovae, Mustelus canis, Alopias vulpinus* and *Isurus oxyrinchus*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 12

MALDI spectra showing peptide mass fingerprints (centre) of *Hexanchus griseus, Negaprion brevirostris, Hemipristis elongata* and *Prionace glauca*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 13

MALDI spectra showing peptide mass fingerprints (top) of modern *Bathytoshia centroura, Raja eglanteria* and *Aetobatus narinari*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 14

MALDI spectra showing peptide mass fingerprints (top) of modern *Rhinoptera bonasus, Hypanus say, Urobatis jamaicensis* and *Hypanus americanus*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 15

MALDI spectra showing peptide mass fingerprints (top) of remaining archaeological vertebra examples; *Aetobatus narinari, Hypanus sabinus, Pristis pristis* and one from a confidently identified *Hypanus sabinus* barb, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURE 16

MALDI spectra showing peptide mass fingerprints (top) of remaining archaeological examples 2 – *Galeocerdo cuvier, Ginglymostoma. cirratum, Rhizoprionodon, Sphyrna tiburo* and *Sphyrna lewini*, with zoomed in sections **(A-D)** in order of increasing *m/z*.

SUPPLEMENTARY FIGURES 17–S51

LC-MS/MS spectra of peptides of potential interest as biomarkers.

SUPPLEMENTARY TABLE 1

Current zooarchaeological results from the Clupper site midden across vertebrate classes.

SUPPLEMENTARY TABLE 2

Modern reference specimens with details.

SUPPLEMENTARY TABLE 3

Archaeological specimens with details.

SUPPLEMENTARY TABLE 4

Local protein sequence database accession numbers.

SUPPLEMENTARY TABLE 5

COL1A1, COL1A2 and COL2A1 matches to *Hypanus sabinus*.

SUPPLEMENTARY TABLE 6

COL1A1, COL1A2 and COL2A1 matches to *Carcharodon carcharias*.

SUPPLEMENTARY TABLE 7

COL1A1, COL1A2 and COL2A1 matches to *Pristis pectinata*.

SUPPLEMENTARY TABLE 8

Selected peptide biomarkers.

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